Short Paper

Phylogenic analysis of serotype Asia1 foot-and-mouth disease virus from Sulaimani/Iraq using VP1 protein: heterogeneity with vaccine strain As1/Shamir/89

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Summary

Foot-and-mouth disease virus (FMDV) serotypes O, A and Asia1 are responsible for a significant number of disease outbreaks in Iraq. The current study can be considered as the first molecular characterization of serotype Asia1 in Iraq. The present investigation reports the detection of serotype FMDV Asia1 from local farms in Sulaimani districts in 2012 and 2014 outbreaks. Phylogenetic analysis of the complete *VP1* gene has shown that FMDV Asia1 field isolates were under genetic novel variant Sindh-08 (group VII) including PAK/iso/11 and TUR/13 strains. The VP1 protein sequence of circulatory FMDV Asia1 genotype showed heterogeneity of nine amino acid substitutions within the G-H loop with the vaccine strain As1/Shamir/89 (JF739177) that is currently used in vaccination program in Iraq. Our result indicated that differences in VP1 protein at G-H loop of the locally circulated FMDV serotype Asia1 strain may be a reason for current vaccination failure.

Key words: Molecular genotyping, Phylogenetic analysis, viral evolution

Introduction

Foot-and-mouth disease (FMD) is a highly contagious and acute vesicular disease impacting domesticated and wild cloven-hoofed animals (Jamal *et al*., 2011; Soleimanjahi *et al*., 2013; Rashid *et al*., 2014). Foot-and-mouth disease is a prototype member of the genus *Aphthovirus* of the family *Picornaviridae* (Ansell *et al*., 1994; Carrillo *et al*., 2005). The virus genome is an 8.5-kilobase, single strand RNA in the plus orientation (Jelokhani-Niaraki *et al*., 2010; Sabar *et al*., 2012). It has four structural proteins known as VP4, VP2, VP3, and VP1. The nucleotide sequences of the VP1 coding region have been used for genetic characterization of FMDV strains because of their significance for virus attachment and entry, protective immunity and serotype specificity (Jamal and Belsham, 2013; Rashid *et al*., 2014). A major highly variable antigenic site in the FMDV is located at the exposed G-H loop, comprising amino acids 134-160 of the capsid protein VP1 (Acharya *et al*., 1989; Logan *et al*., 1993), VP1 plays a significant role in cell infection and is also a primary target for conservative host responses mediated via humoral immunity (Li *et al*., 2011). Foot-and-mouth disease is endemic in Iraq; the first officially recorded instance of FMD strain serotype Asia1 is in 1975 (Ferris and Donaldson, 1992). The purpose of this study is to identify the FMD Asia1 serotype during 2012 and 2014. The second purpose of this study is molecular epidemiology for the detection of FMDV type Asia1 in Sulaimani province for better vaccination.

Materials and Methods

Sampling

Mouth epithelial tissue was collected from suspected FMD infected cattle in two different regions (Halabjay Shahid and Bakrajo) of Sulaimani in 2012 and 2014; the samples were collected by the local veterinary department. The samples were transported to the veterinary laboratory of Sulaimani under cold climate.

RNA extraction

RNA was extracted directly from mouth epithelial tissues by using RNA extraction tissue kit (Genaid, Korea) according to manufacturer's protocol. The extracted RNA was immediately used for cDNA synthesis.

Oligonucleotide primers

Two sets of primers were used in the current study

(Table 1). First the universal primer set (1F/1R) was designed for diagnosis of all FMD virus serotypes (Reid *et al*., 2000; Saeed *et al*., 2011). The second set of primers (As1 1C-505 & NK61) was used to identify FMDV type Asia1 (El-Kholy *et al*., 2007; Saeed *et al*., 2011; Zinnah *et al*., 2012). The primers were synthesized by (Biooner, Republic Korea).

Reverse transcription

Synthesis of first strand cDNA was performed using AccuPower RT premix (Bioneer, Republic Korea). The reaction mixture contained 5 µL extracted RNA template, 1 µL (10 pmole) of IR primer and 1 µL (10 pmole) NK61 primer (Table 1) and 13 µL of ultra-pure water, two reverse primers were added together in the reaction and the mixtures were incubated in a thermocycler (Bio-Rad, USA) programmed at 70°C for 5 min and 4°C for 1 min. The mixture was then added to lyophilize RT premix. The reaction incubated in thermocycler was programmed at 42°C for 60 min, followed by 95°C for 5 min.

Polymerase chain reaction (PCR)

PCR reaction was performed to amplify all serotypes of FMDV. The PCR amplification reaction was carried out in 0.2 ml tubes using AccuPower PCR PreMix. The reaction mixtures contained 5 µL cDNA template, 1 µL (10 pmole) of IF primer, 1 µL (10 pmole) of IR primer, lyophilized master mix and 13 µL of ultra-pure water in 20 µL total volume.

The PCR cycle began with 2 min at 94°C, followed by 38 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min then a final extension at 72°C for 10 min. To amplify FMD type Asia1 (amplicon size of) primer pair of (As1 1C-505 and NK61) with the same conditions apart from 1 min annealing stage were used. To detect all FMD strains and FMD type Asia1, the PCR products of 328 and 911 were electrophoresed in 1% agar, respectively.

Phylogenetic tree

Phylogenetic trees were constructed based on the sequence alignment of *VP1* gene from 48 FMDV strains including Shamir/vaccine strain obtained from the GenBank. The sequence homology and multiple sequences alignment at the nucleotide and amino acid level was performed by the CLUSTALW program (Thompson *et al*., 1994). The phylogenetic tree, based on VP1 sequences was constructed using MEGA 6.0 program employing the neighbor-joining (NJ) method with Kimura 2-parameter nucleotide substitution model (Tamura *et al*., 2013).

Results

Identification serotype Asia1

The representative FMD positive samples were processed with amplification of 328 bp of 5UTR region by PCR, which is a conserved region for all serotypes of FMDV. Asia1 FMD serotypes were detected by amplification of 911 bp by using serotype primer pair (Table 1). The PCR products sequenced were submitted to Genbank with accession number (JX455113 and KM047049), respectively.

Sequence analysis and phylogenetic tree construction

The putative amino acid sequence of serotype Asia1 of two Sulaimani isolates were then compared with twelve other FMDV Asia1 strains (Table 2). Asia1 field isolates genomes exhibited identities ranging from 85.1% to 99%. The highest similarity of (Slemani/IRQ/2012) isolate was found to be with /PAK/iso-1/2011 and TUR/13/2013 with 99% similarity, but the similarity of (Slemani/2/IRQ/2014) isolate with PAK/iso-1/2011 and TUR/13/2013, showed 95% and 94% identity, respectively.

A comparative analysis of the two field isolates exhibited a 95% identity to each other. Interestingly, these isolates presented great amino acid diversity from As1/Shamir vaccine strain ranging from 11-14.9%, respectively.

The complete *VP1* gene of both Slemani sequences were aligned and compared with the reference vaccine strains (As1/Shamir/89, Ind/97, and TAJ/64) for sequence analysis (Fig. 1). VP1 protein revealed (nine, ten and twelve) amino acid substitutions within the G-H loop with the vaccine strains As1/Shamir/89, Ind/97, and TAJ/64 (JF739177, AY687334, FJ785278), respectively.

The phylogenetic tree is shown in (Fig. 2). All the 48 isolates from Asia1 were found to be divided into seven groups (group's I-VII). The phylogenetic analysis of the complete *VP1* gene sequence of FMDV Asia1 and strain in this study showed that two field virus sequences belonged to Sindh-08 (group VII) and were clustered with PAK/iso-1/2011 and TUR/13/2013 isolates. Even though As1/Shamir vaccine strain formed a single group out of seven groups, this result indicates that the vaccine strain that was used in Iraq is producing not homologous with field isolates.

Table 1: List of primers used for detecting FMVV type Asia1 by RT-PCR

Primer name	Sequence	Location on genome	Amplicon	Reference
IF	GCCTGGTCTTTCCAGGTCT	5UTR	328	(15, 16)
IR	CCAGTCCCCTTCTCAGATC			
As1 1C-505-F	TACACTGCTTCTGACGTGGC	1C	911	(18)
NK61_R	GACATGTCCTCCTGCATCTG	2B	911	(15, 17)

Table 2: Identities and divergence of the two isolates from the Sulaimani and 14 references strain

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		93.3	89.0	89.0	85.2	99.0	99.0	87.1	87.6	88.6	88.6	89.0	89.5	91.9	89.5	89.0
2	6.7		85.1	85.1	80.4	92.3	93.3	83.3	84.1	85.6	83.1	85.1	87.1	88.7	86.1	85.6
3	11.0	14.9		94.8	89.0	88.6	89.0	94.3	94.3	94.8	92.4	100	90.0	90.0	92.8	92.3
4	11.0	14.9	5.2		11.4	88.6	89.0	94.3	93.3	94.8	94.8	94.8	89.6	98.1	91.9	91.5
5	14.8	19.6	11.0	88.6		85.2	85.2	86.7	87.2	87.7	87.7	88.5	84.4	86.3	89.6	89.1
6	1.0	7.7	11.4	11.4	14.8		98.1	85.5	87.1	88.1	88.6	88.1	89.5	91.4	89.0	88.5
7	1.0	6.7	11.0	11.0	14.8	1.9		87.1	87.6	88.1	87.6	89.0	89.5	91.9	89.5	89.0
8	12.9	16.7	5.7	5.7	13.3	14.5	12.9		98.1	95.2	92.9	94.3	87.1	88.6	89.0	88.6
9	12.4	15.9	5.7	5.7	12.8	12.9	12.4	1.9		95.7	93.4	94.3	87.2	88.2	88.1	88.6
10	11.4	14.4	5.2	5.2	12.3	11.9	11.9	6.0	4.3		95.7	94.8	90.0	89.1	90.5	90.0
11	11.4	16.9	7.6	5.2	12.3	11.4	12.4	7.1	6.4	4.3		92.4	88.2	87.2	88.2	87.7
12	11.0	15.9	0.0	5.2	11.5	11.9	11.0	5.7	5.7	5.2	7.6		90.0	09.0	92.8	92.3
13	10.5	12.9	10.0	10.4	15.6	10.5	10.5	12.9	12.8	10.0	11.8	10.0		89.6	91.0	90.5
14	8.1	11.3	10.0	10.9	13.7	8.6	8.1	11.4	11.8	10.9	12.8	10.0	10.4		91.5	91.0
15	10.5	13.9	7.2	8.1	10.4	11.0	10.5	11.0	10.9	9.5	11.8	7.2	9.0	8.5		99.5
16	11.0	14.4	7.7	8.5	10.9	11.5	11.0	11.4	11.4	10.0	12.3	7.7	9.5	9.0	0.5	

The upper right values are the percentage identities of the *VP1* gene among viruses and the lower left values are the divergence. 1-2: FMD isolates from the Sulaimani (JX455113 and KM047049), respectively; 3-5: Three vaccines references (JF739177, AY687334, FJ785278), respectively; 6-16: FMD Asia1 isolates from difference countries with GenBank accession numbers (JX435110, KM268898, DQ121402, HQ439195, DQ989309, DQ121113, AY593800, FJ785256, DQ121120, GQ452295, DQ121401), respectively

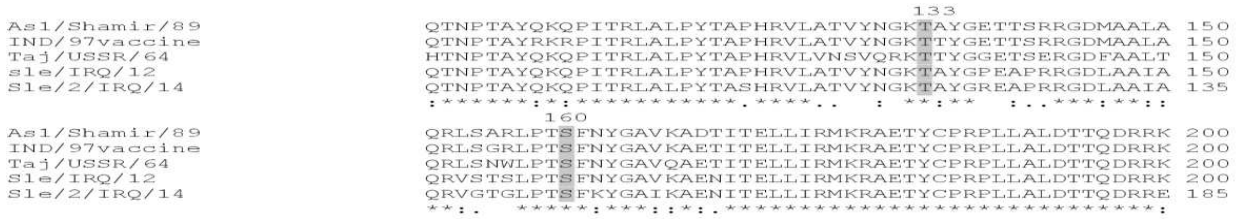

Fig. 1: Two fields isolate sequences alignment with three vaccines reference. Multiple sequences alignment of the amino acid of residue at G-H loop (133-160) of VP1 region two fields isolates FMD type Asia1 with currently available vaccine strain (Shamir89), Ind/97, and TAJ/64

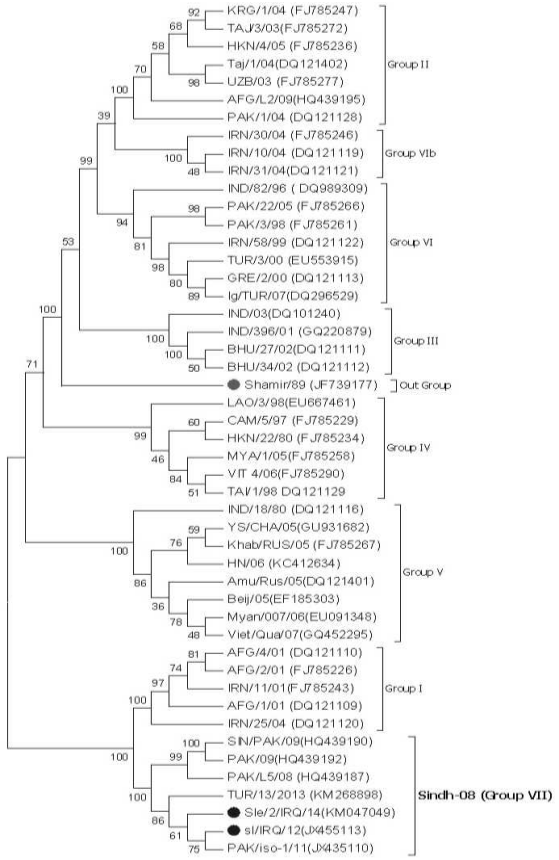

Fig. 2: Neighbor-joining (NJ) phylogenetic tree generated using the nucleotide sequences for the complete VP1 coding regions of serotype Asia1 FMDVs

Discussion

Foot-and-mouth disease virus remains a significant threat to animal husbandry in Iraq. A recent study analyzed molecular and serology detection of serotype Asia1 FMD in Iraq in 2012 (Sabar *et al*., 2012). The phylogenetic study demonstrates that the viruses from new Sindh-08 (group VII) have been responsible for Asia1 FMD outbreaks in the country (Fig. 2). For the first time in Sulaimani/Iraq a thorough study was conducted for the screening of the circulating serotype. Results of this study shed light on the epidemiological situation of type Asia1 FMDV in this region and will help to develop better understanding of evolutionary distribution of the virus. Even though FMD Asia1 appeared in Iraq around 1975 (Ferris and Donaldson, 1992), there is no genetic information about what type of Asia1 FMD existed in the country in previous years that would allow the comparative analysis of the evolution of FMD variants over time. VP1 regions used for comparison in this study are significant for the analysis of serotype specificity, evolution and the occurrence of antigenic heterogeneity, especially in the context of FMD outbreak (Ruiz-Jarabo *et al*., 2000; Domingo *et al*., 2003). The As1/Shamir vaccine strain exhibited high levels of genetic diversity (14.9-11%), and low identity (85.1-89%) with field isolates most likely be reflected in antigenic differences. The antigenic analysis reported by the WRL, Pirbright, UK shows that a virus As/PAK/29/2009 which was in the group Sindh-08

(group VII) along with both Sulaimani FMDV Asia1 isolates, was not neutralized antisera generated against the Asia1/Shamir vaccine (Jamal *et al*., 2011). Nevertheless, Shamir/89 vaccine strain is considered as an appropriate one of the best choice strains for induction in vaccine when compared to the other two vaccine strains (Ind/97 and TAJ/64) [Fig. 2 and Table 2]. Similar investigations are continuously required in the disease-endemic areas of Iraq for the improvement of vaccination and disease control strategies to avoid the emergence of new outbreaks with distinct variants of the virus. National effort must be made to provide an alternate vaccine strain instead of Shamir/89 vaccine strain that has less diversity and better antigenic matching with Asia1 field strain, in order to provide better control measure.

Despite meaningful information being available about the virus, the disease, and vaccines, FMDV remains a major threat to the livestock industry worldwide. Moreover, molecular characterization of FMDV Slemani Asia1 isolates showed considerable diversity with As1/Shamir/89 vaccine strain. Outbreaks in vaccinated animals represent challenges with regard to evaluation of the existing vaccine strain and identification of new correlate vaccine strain which is relevant to Iraq.

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